Intrathecal Immune Responses in Stiff Person Syndrome and Multiple Sclerosis

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Doctoral thesis by

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List of abbreviations

ALCAM	activated leukocyte cell adhesion molecule
APC	antigen presenting cell
BAFF	B cell activating factor
BBB	blood-brain barrier
BCR	B cell receptor
Bmax	binding capacity
CIS	clinically isolated syndrome
CCL / CXCL	chemokine ligand
CCR / CXCR	chemokine receptor
CD	cluster of differentiation
CNS	central nervous system
CSF	cerebrospinal fluid
D	diversity
DC	dendritic cell
DMSO	dimethyl sulfoxide
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein Barr virus
E. coli	*Escherichia coli*
GA	glatiramer acetate
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GC	germinal centre
HLA	human leukocyte antigen
HSV-1	Herpes Simplex virus type-1
ICAM / VCAM	intracellular adhesion molecule / vascular cell adhesion molecule
IEF	isoelectric focusing

IFN	interferon
Ig	immunoglobulin
IL	interleukin
J	joining
kDa	kilo Dalton
MeV	Measles virus
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MS	multiple sclerosis
NLR	nucleotide oligomerisation domain-like-receptor
NOD	non-obese diabetic
OCBs	oligoclonal bands
PBMC	peripheral blood mononuclear cell
PER	progressive encephalomyelitis with rigidity
PPH	post puncture headache
RA	rheumatoid arthritis
rh	recombinant human
RIA	radioimmunoassay
SLE	systemic lupus erythematosus
SPS	stiff person syndrome
T1D	type 1 diabetes
TCR	T cell receptor
TGF	transforming growth factor
T_H	T-helper
TLR	Toll-like receptor
TNF	tumour necrosis factor
V	variable
VGKC	voltage-gated potassium channel
VZV	Varizella Zoster virus

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Paper I

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Paper II

Skorstad G, Hestvik AL, Torjesen P, Alvik K, Vartdal F, Vandvik B and Holmøy T. GAD65 IgG autoantibodies in stiff person syndrome: clonality, avidity and persistence. *European Journal of Neurology* 2008; 15: 973-80.

Paper III

Skorstad G, Hestvik AL, Vartdal F and Holmøy T. Cerebrospinal fluid T cell responses against glutamic acid decarboxylase 65 in patients with stiff person syndrome. *Journal of Autoimmunity* 2008; 32: 24-32.

Papers related to the thesis:

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Hestvik AL, *Skorstad G*, Price DA, Vartdal F and Holmøy T. Multiple sclerosis: glatiramer acetate induces anti-inflammatory T cells in the cerebrospinal fluid. *Multiple Sclerosis* 2008; 14: 749-758.

Basic concepts in immunology

The immune system has evolved to recognise and combat infectious agents. This is accomplished through the immune system's ability to distinguish between self and non-self. The protection is achieved through the highly coordinated action of the innate and adaptive arms of the immune system (Murphy 2008).

The *innate* branch of the immune system is the first line of defence against microbial agents. Receptors on the innate arm of the immune system are evolutionary conserved germline-encoded proteins which include Toll-like receptors (TLR) and nucleotide oligomerisation domain-like-receptors (NLR). TLRs which are receptors localised either at the cell surface or within endosomes, recognise a bewildering range of conserved microbial structures, such as bacterial cell wall components, lipoproteins, highly conserved microbial proteins, and bacterial and viral nucleic acids, often referred to as pathogen-associated molecular patterns or danger-associated molecular patterns (Janeway 2002; Kawai & Akira 2005). By contrast, the NLRs are a family of intracellular sensors that in addition to detect conserved microbial structures also sense "danger signals". These molecules alert the immune system upon recognition of dangerous environmental events, maybe independently of a microbial trigger (Fritz 2006). These relatively non-specific receptors are expressed on a variety of innate immune cells such as granulocytes, dendritic cells (DC), macrophages and natural killer cells. The innate immune system responds rapidly (within few hours) after onset of an infection, compared to days required for the mobilisation of the adaptive immune system.

Adaptive immunity, also known as acquired immunity, is the specific response of lymphocytes to an antigen, including the development of immunological memory.

Immunological memory is known to persist for a lifetime and therefore carry the "history of the individual's adaptive immune responses" (Crotty 2003; Amanna 2007). The adaptive immune response is generated by clonal selection and expansion of lymphocytes. The key players within the adaptive immunity are T cells, B cells, and professional antigen presenting cells (APC) including DCs, macrophages and B cells. The professional APCs are important in connecting the innate and the adaptive arm of the immune system by integrating innate information and conveying it to the T and B lymphocytes.

The adaptive immune response

Self - tolerance mechanisms

Our immune system is the body's sixth sense (Goodnow 2005). The mammalian immune system has an extraordinary capability for generating a huge receptor diversity that can mount a response to almost any chemical structure entering the body. The receptors that are involved in this "great deed" are the T cell receptors (TCR) displayed on T cells and the immunoglobulin (Ig) expressed on the surface of B cells as B cell receptors (BCR). Virtually unlimited receptor diversity can be generated in mammals by the process of variable (V), diversity (D), joining (J) V(D)J recombination that occur selectively in lymphocytes. During T- and B- cell differentiation which takes place in the central lymphoid tissues, three separate gene segments, the V, D and J are assembled by V(D)J recombination into unique TCR and BCR genes. Random addition and deletion of nucleotides at the junctions between gene segments further contribute to the diversity of the third hypervariable region. During the late phase of the immune response in the peripheral lymphoid tissues, somatic hypermutation

substitutes single nucleotides of BCR genes leading to the production of high-affinity antibodies (Goodnow 2005; Murphy 2008).

Between 20 and 50 % of TCRs and BCRs generated by V(D)J recombination bind to self antigens (Zerrahn 1997; Wardemann 2003). However, the immune system has developed several checkpoints to prevent self-antigens from triggering self-reactive B and T lymphocytes. The cellular strategies engaged to deal with self-reactive receptors include: 1) clonal deletion; a cell bearing a "forbidden" receptor can be triggered to die according to Burnet's concept of clonal selection, 2) receptor edition; further V(D)J recombination or somatic hypermutation of the aberrant receptor giving rise to a new receptor that is not self reactive, 3) "clonal anergy or tuning"; changes in gene expression can reduce the ability of the cell to be triggered by self-reactive receptors, and 4) extrinsic control mechanisms that limit the supply of essential growth factors, co-stimuli and other necessary factors including suppression by regulatory T cells or regulatory B cells (Burnet 1961; Kronenberg & Rudensky 2005; Fillatreau 2002; Hu 2007). The result of these processes is that most lymphocytes expressing self-reactive receptors dangerous for the host are eliminated.

T cell immunity

Progenitor T cells migrate from bone marrow to the thymus where further maturation and selection processes occur. Within the cortical and medullar compartments of the thymus, T cells undergo positive and negative selection driven by the interactions with thymic epithelial cells that display composites of self peptides and major histocompatibility complexes (MHC) molecules on their cell-surface (Starr 2003). T cells are divided into two major subsets identified by the expression of the unique functional cluster of differentiation (CD) surface

molecules, denoted CD4 and CD8. $CD4^+$ T cells recognise peptides presented on MHC class II, whereas $CD8^+$ T cells recognise peptides presented by MHC class I.

Mature T cells leaving the thymus to enter the circulation are termed naïve. They circulate in the lymphatic system, through secondary lymphoid organs where they can interact with APCs, in principal DCs, but also macrophages and B cells. DCs which are found in the T cell areas of the lymphoid tissues are the most potent stimulators of T cells, and are believed to be the main cells that can activate naïve T cells (Banchereau & Steinman, 1998).

The MHC complex

The genes in the MHC complex

Molecules of the MHC were first discovered for their importance in rejection of tissue grafts between genetically non-compatible individuals (Thorsby 2009). The MHC, named the human leukocyte antigen (HLA) complex in humans, is encoded by a highly polymorphic complex of genes contained within 4 megabase pairs (1 % of the genome) on chromosome 6p21.3. The MHC region is divided into three; the class I gene region, followed by the class II and the class III gene region. Several of the genes encode classical immune response molecules, central in antigen presentation. The HLA genes are highly polymorphic and the genes are located in the class I (HLA -A, -C, -B) and class II (HLA -DR, -DQ, -DP) regions.

The MHC molecules

The function of MHC molecules is to bind peptides and display them on the cell surface to T cells. These membrane glycoproteins are divided into two types: Whilst MHC class I molecules are expressed on all nucleated cells, the MHC class II molecules are expressed primarily on professional APCs.

Whereas the MHC class I molecules are composed of a heterodimer of a 43 kilo Dalton (kDa) membrane-spanning α-chain bound non-covalently to a 12 kDa β2-microglobulin (which does not span the membrane), the MHC class II molecules are composed of two transmembrane non-covalently associated glycoprotein chains, the 33 kDa α-chain and the 28 kDa β-chain (Murphy 2008). Whereas the MHC class I peptide-binding groove is constrained allowing only short peptides of 8 - 10 amino acids to bind, the ends of the MHC class II peptide-binding groove are open, allowing peptides of unlimited length to bind. Peptides eluted from MHC class II molecules are normally between 10 and 34 amino acids long (Murphy 2008; Madden 1995; Rudensky 1991; Vartdal 1996).

Presentation of T cell epitopes

$CD8^+$ T cells recognise peptides derived from cytosolic proteins in the context of MHC class I molecules, whereas $CD4^+$ T cells interact with MHC class II molecules that presents fragments of proteins that have been degraded in the endocytic and phagocytic vesicles (Watts 1997).

The antigen fragments that bind to MHC class I are usually derived from endogenous proteins synthesised within the cell, including viral proteins, after being transported from the cytosol by proteins in the endoplasmatic reticulum membrane (Murphy 2008). Furthermore, in addition to sampling of endogenous peptides for presentation by MHC class I molecules, DCs and macrophages have the capability to present exogenous antigens internalised via the endocytic pathway to $CD8^+$ T cells (Ackerman 2004). Such “cross-presentation” is thought to be critical for initiating $CD8^+$ T cell responses to antigens that would not otherwise gain access to the MHC class I presentation pathway in DCs.

Protein antigens determined for MHC class II molecules entering the endocytic pathway are unfolded and degraded into peptides before reassembling as integral components of the mature class II complex (Jensen 1995). In the different endosomal / lysosomal compartments, processing of protein antigens in the MHC class II pathway is achieved by exopeptidases, endopeptidases, and γ-interferon-induced lysosomal thiol reductase, including several cysteine proteases. The outcome of antigen processing is thought to be a key determinant of the quality and quantity of a $CD4^+$ T cell response (Watts 2004).

T cell activation and their effector functions

TCR interaction with the peptide / MHC complex is necessary, but not sufficient for triggering of the T cell. To become fully activated, T cells, and in particular naïve cells, must engage co-stimulatory molecules. The B7 molecules CD80 and CD86 displayed on the surface of APCs are recognised by CD28 molecules expressed by T cells. The fate of the T cell; death, fully activated or tolerant, depends on the strength and length of the interaction with peptide / MHC complex and cytokines in the environment (Lanzavecchia & Sallusto 2001; Sallusto & Lanzavecchia 2001). Accordingly, if the T cell receives appropriate co-stimulation, it will divide and differentiate into long-lived memory cells or effector cells.

Triggered $CD4^+$ effector T cells can differentiate into separate functional subsets, and exert their effector functions mainly through cytokine secretion. More than 20 years ago, Mosmann and Coffman described in mice the presence of two distinct populations of $CD4^+$ T-helper (T_H) cells, T_H1 and T_H2, characterised by their distinct cytokine profile (Mosmann & Coffman 1989). Studies on T_H1 and T_H2 cells in humans have been more challenging, since a high percentage of $CD4^+$ T cells isolated from blood of healthy individuals have been found to display a mixed cytokine profile upon activation. Nevertheless, naïve conventional

$CD4^+$ T cells have five (and possibly more) distinct fates determined by signal patterns received through initial interaction with the antigen. These five T_H cell populations include: T_H1 cells with interferon (IFN)-γ as their signature cytokine, T_H2 cells producing predominantly the signature cytokines IL-4, IL-5, and IL-10, T_H17 cells secreting IL-17, IL-22 and transforming growth factor-β (TGF-β), and the recently identified skin-homing helper T cell subset, T_H22 cells, that secrete IL-22 but neither IL-17 nor IFN-γ. In addition, adaptive regulatory T cells producing TGF-β and IL-10 are responsible for suppression of other T cells (Zhu & Paul 2008; Duhen 2009; Trifari 2009). Notably, it was recently demonstrated that T_H cell lineages possess an unexpected degree of plasticity, which may allow them to adopt alternative fates or to acquire functions usually restricted to an opposite $CD4^+$ T cell linage (Wei 2009; Lee 2009).

Although no such subset diversification exists among $CD8^+$ T cells a variety of effector functions have been described, including cytotoxicity and cytokine production (Williams & Bevan 2006). Whilst some of these effector cells respond with only a limited functional repertoire, other can exert several effector functions such as production of IFN-γ, IL-2 and tumour necrosis factor (TNF)-α (Stemberger 2007).

B cell immunity

In mammals, B cells arise from hematopoietic stem cells in the bone marrow where they acquire a unique BCR. B cells mature independently of antigen stimulation into pro-B cells, and then further into pre-B cells and immature B cells before entering the antigen-dependent phase in the peripheral lymphoid tissues (Browning 2006).

Activation of a naïve B cell is initiated in response to specific antigen binding to the BCR. Following antigenic stimulation, B cells can process antigen and present it on their MHC II molecules to antigen-specific $CD4^+$ T cells, which give cognate help to the B cell (Lanzavecchia 1985). At the immunological synapse, antigen specific $CD4^+$ T cells are activated and successively stimulate B cells through CD40L-CD40 interactions and cytokine secretion (Banchereau 1994). Optimal activation of human naïve B cells requires a third signal that can be delivered by TLR agonists or by cytokines produced by activated DCs (Ruprecht & Lanzavecchia 2006). Activated B cells can then adopt one of two fates: 1) movement into extrafollicular areas to become short-lived antibody producing cells, or 2) movement into B cell follicles and establishment of GCs (Jacob 1991). After affinity maturation in GCs, both long-lived memory B cells competent to give rapid and enhanced response to secondary antigen challenge and plasma cells capable of producing high-affinity antibodies are formed (Rajewsky 1996). From the GCs, long-lived plasma blasts migrate to the bone marrow where they differentiate into long-live plasma cells, while activated memory B cells may migrate to inflamed tissues and also to other organs including the brain (Knopf 1998; Dalakas 2008).

The TNF family members B cell activating factor (BAFF) and a proliferation-inducing ligand are vital factors for B cells survival, differentiation, formation of GC and production of Ig (Mackay & Schneider 2009). BAFF receptors are displayed on all B cells from stem cells to plasma cells (Dalakas 2008). High levels of BAFF are associated with the development of autoimmune disorders in animal models, and an excess of BAFF has been found in serum of patients with diverse autoimmune conditions (Mackay & Schneider 2009).

B cells also have a role in presenting antigen to $CD4^+$ T cells. B cells are adapted to bind specific soluble molecules through their cell-surface Ig and to internalise bound molecules by

receptor-mediated endocytosis. Since B cells constitutively express high levels of MHC class II molecules, high levels of specific peptide-MHC class II complexes appear on the B cell surface (Murphy 2008). Moreover, it has been demonstrated that receptor mediated uptake of antigen makes B cells 100 – 1.000 times more potent on presentation of specific antigen compared to other APCs, and they are therefore especially effective at presenting low concentrations of antigens bound by their BCR (Lanzavecchia 1990).

Optimisation of antibody affinity is a hallmark of the humoral immune response that takes place in hundreds of transient microstructures called GCs. The term "GC" was coined more than 120 years ago by Walter Flemming, who observed accumulations of large lymphocytes undergoing mitosis in the follicles of lymph nodes and proposed these to be the major source of all lymphocytes in the body (Nieuwenhuis & Opstelten 1984). Although it is not Flemming's GCs but the bone marrow which is the site of lymphocyte generation, the GCs are now known to be associated with T cell dependent antibody responses and are the most important sites for generation of high-affinity B cells. Furthermore, affinity maturation is often seen in parallel with an increased concentration of specific antibodies and class switch from IgM to other isoforms (Griffiths 1984; Siekevitz 1987; Chien 1988). Positively selected GC B cells may differentiate into either memory B cells or antibody-secreting plasma cells that subsequently exit GCs to become part of the circulating lymphocyte pool or migrate to the bone marrow as long-lived plasma cells, respectively (MacLennan 1994).

Immunoglobulin structure

Igs are the secreted form of the BCRs. Igs are large molecules of approximately 150 kDa, and are composed of two different kinds of polypeptide chains: heavy chains and light chains. Two heavy chains are linked to each other by disulfide bonds and each light chain is linked to a heavy chain by disulfide bonds and noncovalent interactions. This gives rise to an antibody molecule with two identical antigen-binding sites. Each of the four chains has a V region at its amino terminus, and this region contributes to the antigen-binding site. X-ray crystallography of antigen : antibody complexes has demonstrated that localised regions of hypervariable sequences of the Ig V regions form the antigenic-binding site of the antibody. The carboxyl terminal region, named the constant region, comprises one of five heavy chain classes or isoforms (IgM, IgD, IgG, IgA and IgE), some of which are found to have several subtypes. The constant regions are responsible for the biological effector functions of the antibody (Murphy 2008).

Autoimmunity

Autoimmunity can be defined as adaptive immunity specific for self antigens (Murphy 2008). An overactive autoimmune response is believed to be one possible mechanism underlying development of autoimmune diseases, which affect 3 - 5 % of the human population in the industrialised part of the world (Ashwood 2006). Autoimmune disease can be divided into either organ-specific disorders, such as Type 1 diabetes (T1D) and myasthenia gravis, or systemic illnesses, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).

Little is known about how and why autoimmunity is triggered. A widely accepted hypothesis is that autoimmune diseases arise from an unfortunate combination of genetic and environmental factors. The low concordance rate for many autoimmune diseases among monozygotic twins suggests a substantial involvement of environmental factors. Evidence has indicated that microbes can initiate, enhance, or, conversely, abrogate autoimmunity (Christen 2005). Thus, a number of mechanisms for infection-induced autoimmunity have been postulated: 1) infection of target cells and organs, resulting in tissue destruction that may cause the release of sequestered antigens and enhanced antigen presentation by DCs and macrophages (under inflammatory conditions, upregulation of antigen processing events may lead to enhanced presentation of previously cryptic epitopes by the APCs which can be presented to self-reactive T cells), 2) epitope mimicry, and 3) bystander activation (Lehmann 1992; Benoist & Mathis 2001).

The involvement of activated B cells in autoimmune diseases has traditionally been viewed as a secondary consequence of the breakdown of T-cell tolerance (Dalakas 2008). This does not exclude that B cell play an instrumental role in the disease process. Thus, it was early demonstrated by

transfer experiments that some human diseases, such as myasthenia gravis, are directly mediated by pathogenic antibodies (Toyka 1975; 1977). In recent years, experimental evidence supporting an even more pronounced and diverse role of B cells in the pathogenesis of autoimmune diseases has grown: Thus, B cells could have a direct role via 1) production of autoantibodies or cytokine secretion, 2) indirectly influence T cell responses by presenting antigen or 3) by secreting antibodies that bind to antigens to form immune complexes. The observation that B cell depletion by using monoclonal antibodies against CD20 such as Rituximab, which depletes all cells of the B cell lineage except from stem cells, pro-B cells and plasma cells, as an effective therapy in autoimmune diseases such as RA and multiple sclerosis (MS), has provided an increased drive to explore the functions of B cells in autoimmune diseases (Edwards & Cambridge 2006; Hauser 2008).

Immune surveillance of the central nervous system

The concept of immune privilege of the central nervous system (CNS) emerged from studies of a phenomenon that had been observed for over 130 years, namely that allografts, which are rapidly rejected from tissues such as skin, are accepted when placed in the brain. As no immune rejection was evoked, the organ was called “privileged” (Medawar 1948). However, more recent studies have shown a slow and inefficient clearance after inoculation of virus into the parenchyma of the CNS (Stevenson 2002). Consequently, the claim that the CNS is a site of immune privilege has been modified, and it is now rather viewed as an immunologically specialised site (Ransohoff 2003).

Lymphocytes are rare in the CNS of healthy individuals partly because of the blood-brain barrier (BBB), which is a protective barricade that limits the entry of large molecules and circulating immune cells into the CNS. Immune activation is also limited in the CNS owing to the lack of endogenous APCs and a relative lack of lymphatic drainage of the parenchyma. To be able to interact with immune-competent cells in secondary lymphoid organs outside the BBB, the mammalian CNS has evolved pathways for delivery of antigens from the CNS parenchyma to the peripheral circulation. Additionally, three or more routes have been suggested to exist for leukocyte migration into the CNS from blood; 1) from the blood to the cerebrospinal fluid (CSF) via the choroid plexus within the ventricles of the CNS, 2) from the blood to the subarachnoid space and, 3) from the blood to the parenchymal perivascular space (Harling-Berg 1999; Ransohoff 2003).

Under physiological conditions lymphocyte traffic across the BBB has been observed to be very low (Gowerman 2009). Pioneering studies demonstrated that the BBB strictly controls T cell traffic into the CNS as only activated but not resting T cells were able to

penetrate this barrier in healthy experimental animals (Wekerle 1987; Hicky 1991). Activated $CD4^+$ memory T cells enter CNS directly from the systemic circulation to ′inspect′ the subarachnoid space, retaining their capability to either initiate local immune reactions or return to secondary lymphoid organs (Kivisakk 2003; 2009). Yet, how autoreactive T cells gain entry to an uninflamed brain to initiate disease has been unknown. Intriguing, it has recently been demonstrated that autoreactive T_H17 cells expressing the chemokine receptor (CCR) 6 can gain access to the uninflamed CNS trough a "chink in the armour" of the BBB; the choroid plexus, by interaction with the chemokine ligand (CCL) 20 (Reboldi 2009; Axtell & Steinman 2009). CCL20 is constitutively expressed by epithelial cells of the choroid plexus in mice and humans, and it was suggested that the CCR6 - CCL20 axis controls an evolutionary conserved pathway of immune surveillance of the CNS (Reboldi 2009). Although huge numbers of immune cells can directly enter the CNS parenchyma through the "leaky" BBB during inflammation, lymphocyte recruitment into the CNS during MS or experimental autoimmune encephalomyelitis (EAE) is not random as T cells detected in the CNS parenchyma and in the CSF are phenotypically distinct from T cells found in the blood (Engelhardt 1998; Zeine & Owens 1992; Hestvik 2008).

Leukocyte migration into the CNS parenchyma is a multi-step process: first, the cells must cross the vascular endothelial wall, and second they must transverse the astrocyte lining, each with its own basement membrane. The attachment of blood-borne T cells to the vascular bed, followed by diapedesis of immune cells across the endothelial barrier is mediated by several adhesion molecules, including intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), α4-integrins and laminins expressed on inflamed BBB (Bullard 2007; Carman & Springer 2004; Engelhardt 2005). New data has shown that the activated leukocyte cell adhesion molecule (ALCAM) which bind to CD6 on leukocytes,

replaces VCAM-1 in the BBB endothelium during the development of neuroinflammatory diseases such as MS (Cayrol 2008). Blocking of ALCAM suppressed transmigration of T cells through the BBB and ameliorated EAE. Inflammatory chemokines direct leukocyte trafficking to inflamed tissues “on demand”; for example the chemokines CCL5 (RANTES), CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL10 (IP-10) attract mainly monocytes and T cells, which have been associated with lesion development in MS (Farina 2005).

To reach the brain parenchyma leukocytes must traverse the parenchymal basement membrane and the glial limitans. Recent evidence has shown that macrophage-derived gelatinase matrix metalloproteinase (MMP)-2 and MMP-9 activity is crucial for leukocyte penetration of the glia limitans during EAE (Agrawal 2006). Ablation of both MMP-2 and MMP-9 in double knockout mice confers resistance to EAE by preventing leukocyte infiltration into the CNS (Agrawal 2006).

The mechanisms of B cell migration into the CNS are not well understood as those that apply for T cells. However, the chemokine molecules CXCL13, CXCL10 and CXCL12 that are secreted from the endothelial wall are up-regulated in the brain of MS patients, thereby facilitating the recruitment and transmigration of B cells into the brain (Ritchie 2004; Meinl 2006). Additionally, B cell migration into the CNS is also facilitated by binding of the adhesion molecules very late antigen-4 and lymphocyte function-associated antigen-1 to their counter-receptors VCAM-1 and ICAM-1 on the endothelial cells (Alter 2003).

Stiff Person Syndrome

Historical background

Stiff man syndrome was originally described in 1956 by Moersch and Woltman (Moersch & Woltman 1956). Their first case in 1924 was a 49-year-old male farmer from Iowa who was examined because of a complaint of "muscle stiffness and difficulty in walking", hence the original term "stiff man syndrome". The pioneers went on and after 32 (from 1924 to 1956) years with seeking they published a series of 14 (10 males and 4 women) other similar patients (Moersch & Woltman 1956). Since then the disease has been observed more frequently in women than men, and is now generally referred to as Stiff Person Syndrome (SPS) (Goetz 1983). SPS is a rare disorder of the CNS with a reported annual incidence of one per million in the European population, which could be an underestimate due to misdiagnosis as a psychiatric disorder (Koerner 2004).

Clinical presentation

The core clinical features of classical SPS are stiffness that is prominent in axial muscles with co-contraction of agonist and antagonist muscles, and sudden episodic spasms in the absence of another disease that causes similar symptoms (Moersch & Woltman 1956; Levy 1999). SPS usually appears in adulthood and generally has a fluctuating, slowly progressive course; however, in a few cases sudden death has been reported (Mitsumoto 1991). The SPS spectrum which include stiff limb syndrome, jerking SPS and progressive encephalomyelitis with rigidity (PER) share clinical, laboratory, electrodiagnostic and histopathological features

(Brown 1997; Leigh 1980; Whiteley 1976). Some patients present initially with stiff limb syndrome progress over time to classical SPS and from that to PER (Duddy & Baker 2009). In 5 % of the patients, SPS is a paraneoplastic manifestation. Such patients have been reported to have more prominent stiffness in neck and arms than other SPS patients (De Camilli 1993; Butler 2000).

Continuous co-contraction of agonist and antagonist muscles as a result of involuntary firing of motor units at rest is central to the pathophysiology of SPS (Lorish 1989; Thompson 2001). Because γ-aminobutyric acid (GABA) is the brain's predominant inhibitory neurotransmitter, GABAergic neurotransmission could play a role in the pathogenesis. Moreover, *in vivo* magnetic resonance spectroscopy has revealed a prominent and significant reduction in GABA levels in the motor cortex but not in the occipital cortex of SPS patients (Levy 2005). This is consistent with a recent paper describing changes in central GABA-A receptors linked benzodiazepine binding sites in selected brain regions on positron emission tomography scans (Perani 2007). These data strongly suggest a GABAergic deficit in SPS, but it remains unclear whether this is due to a functional block or reflects primarily neuronal loss.

The histological findings of the few autopsies carried out so far have been inconsistent. The first report from Moersch and Woltman failed to demonstrate pathological lesions of specific areas of the CNS (Moersch & Woltman 1956). Evidence for an inflammatory process with selective loss of GABAergic neurons within the cerebellum and spinal cord, or a more aggressive inflammatory picture of perivascular lymphocytic infiltration including gliosis within the spinal cord, cerebral cortex, brainstem and basal ganglia have later been reported in some patients with SPS (Warich-Kirches 1997; Meinck 1994; Mitsumoto 1991). Furthermore, autopsy has revealed vacuolar degeneration of anterior horn cells at the lumbar

segments of the spinal cord associated with prominent microglia proliferation and evidence of discrete infiltration of $CD8^+$ cytotoxic T cells in patients with atypical SPS (Saiz 1999).

Glutamic Acid Decarboxylase

Glutamic acid decarboxylase (GAD) converts glutamate to GABA (Erdo & Wolff 1990; Lernmark 1996). The two isoforms of GAD are named according to their respective molecular weights, GAD65 (585 amino acid residues) and GAD67 (594 amino acid residues). The isoforms are encoded by two different genes localised on chromosome 10 and 2 respectively (Brilliant 1990; Karlsen 1991; Bu & Tobin 1994; Erlander 1991). Although the two isoforms display a high similarity in their amino acid sequences, differences are found in their interaction with the co-factor pyridoxal-5′-phosphate and in enzyme kinetics for GABA production (Battaglioli 2003). Whereas cytosolic GAD67 is found to be constitutively active and is responsible for basal levels of GABA, the inducible cytosolic GAD65 associated with the GABA vesicle membrane is mostly present without its co-factor as an autoinactivated apoenzyme (Fenalti 2007). Upon reactivation with its co-factor, apo-GAD65 becomes holo-GAD65 that can catalyze synthesis of GABA when additional neurotransmitter is needed (Kash 1997). Whilst both GAD isoforms are found in the brain, only GAD65 is found in pancreas, though its function there is not clear (Lernmark 1996).

GAD65 IgG antibodies and GAD65-specific T cells in SPS

GAD IgG antibodies were first reported in 1988 by Solimena and co-workers in a patient affected by SPS and epilepsy. The GAD65 isoform was identified as the predominant autoantigen (Solimena 1988; Butler 1993). Antibodies to GAD65 are also found in 80 % of

patients with T1D and in some patients with cerebellar ataxia, epilepsy, myoclonus, Batten disease and Grave's disease (Baekkeskov 1990; Saiz 1997). While antibodies against GAD65 in T1D target mainly conformational epitopes, the GAD65 IgG antibodies in SPS have been shown to be of significantly higher levels and to detect both linear and conformational epitopes (Kim 1994; Bjork 1994; Daw 1996). The observation that disease severity in SPS did not correlate with GAD65 IgG antibody levels in serum or CSF in SPS patients suggested that GAD65 IgG antibodies may not have a direct pathogenic role in SPS (Rakocevic 2004; Burns 2005). Also, GAD65 IgG antibodies do not seem to transfer the disease symptoms from mother to infants (Nemni 2004). As GAD65 is a cytoplasmic molecule the pathogenic role of GAD65 IgG antibodies has been questioned. However, intrathecal administration of IgG from sera of patients with GAD65 IgG antibody-associated SPS induced neurophysiological and biochemical SPS-correlates in rats (Manto 2007). This phenomenon was not observed after administration of IgG antibodies from T1D patients, which indicate that SPS could be the direct consequence of antibody-mediated neuronal dysfunction (Manto 2007). Also, electrophysiological studies have shown that CSF or serum positive for GAD65 IgG antibodies from SPS patients, and not from T1D patients, can reversibly inhibit GABAergic transmission in rat cerebellar slices (Dinkel 1998; Ishida 1999; Vianello 2008).

Additional evidence for B cell involvement in SPS has been demonstrated by the beneficial effects of intravenous Ig (Vasconcelos 2003). It has also been observed that some SPS patients respond to treatment with immunomodulatory agents (Vicari 1989; Brashear & Phillips 1991; Hao 1999; Dalakas 2001). Plasmapheresis has been reported to be beneficial in some SPS patients, and in a case study the improvement of symptoms correlated with decreased GAD65 antibody levels (Dalakas 2009). Finally, anti-CD20 B cell therapy has been shown to be beneficial in reducing stiffness and increasing mobility, and resulted in

disappearance of GAD65 IgG antibodies in patients with PER and SPS (Saidha 2008; Baker 2005).

Attempts to detect GAD65-specific T cells in the peripheral blood from SPS patients have been few and not very successful, despite the fact that class-switched and high-affinity antibodies to GAD65 imply T cell involvement. Nevertheless, peripheral blood mononuclear cells (PBMCs) from some SPS patients have been shown to respond weakly to synthetic GAD65 peptides or to recombinant human (rh)GAD65 (Costa 2002; Hummel 1998; Lohmann 2000, 2003). Additionally, a GAD65-specific T cell line has been established from the blood of an SPS patient, even though no primary proliferative PBMCs response against GAD65 was detected in the patient (Schloot 1999).

Other aspects of autoimmunity in SPS

A striking association with other organ-specific autoimmune diseases has been described in SPS (Solimena 1990). T1D is relatively common in SPS and occur in approximately 30 - 60 % of the patients, whereas the converse is not true (Solimena 1990; 1991). Other autoimmune diseases less commonly associated with SPS include thyroid diseases, pernicious anaemia, vitiligo and Graves' disease (Solimena 1990). Furthermore, it was recently reported of an SPS patient with celiac disease and dermatitis herpetiformis (O'Sullivan 2009). In addition, antibodies against GABA receptor-associated protein, a protein involved in the trafficking and assembly of the GABA-A receptor has been found in 70 % of SPS cases in one study (Raju 2006). Also, an antibody to glycine receptors has been identified in a patient with PER (Hutchinson 2008). In paraneoplastic variants SPS, antibodies are directed against two other proteins in the GABAergic and glycinergic

synapses; amphiphysin and gephyrin (Folli 1993; De Camilli 1993; Butler 2000). Interestingly, injection of serum IgG antibodies from a patient with paraneoplastic SPS and antibodies to amphiphysin into rats resulted in transient symptoms of stiffness with spasms resembling human SPS, showing that the IgG antibodies could be pathogenic (Sommer 2005). Recent studies from the same group showed that anti-amphiphysin antibodies from the same patient reduced GABA-induced calcium influx in embryonic motor neurons (Geis 2009).

An association to HLA-DQB1*0201 and HLA-DRB1*0301 in SPS has been reported in two studies (Pugliese 1993; Dalakas 2000). However, it is important to note that these studies were carried out on relatively small populations. In a short familial report on a father and his daughter who both had SPS associated with GAD65 IgG antibodies, the HLA-DQB1*0201 (father) and DRB1*0301 (daughter) alleles did not segregate with SPS (Burns 2003). Interestingly, HLA-DRB1*06, which is an allele that is extremely uncommon in T1D patients, has been reported to be associated with a lower co-occurrence of diabetes in SPS (Pugliese 1993).

Multiple Sclerosis

MS is an inflammatory disorder of the brain and spinal cord, affecting more than 1 in 1.000 Caucasians living in temperate climate (Pugliatti 2002). In most patients the disease starts with transient neurological symptoms that are denoted clinically isolated syndrome (CIS), whereas an MS diagnosis requires further radiological or clinical dissemination of the disease process (McDonald 2001). It is thought that MS occurs in individuals with complex genetic-risk profiles after exposure to an environmental trigger that activates auto-reactive T cells allowing them to migrate across the BBB. In animal models of the inflammatory aspect of MS, T cells reactivated by CNS-resident APCs presenting CNS antigen can then recruit innate immune cells, which are important actors in the demyelination and neurodegeneration processes (McFarland 2007; Compston 2008). The hallmark features of MS are inflammatory demyelinating plaques with partial axonal preservation and reactive gliosis in the brain and spinal cord, particularly during the early (relapsing) stage of the disease (Prineas 1984; Kutzelnigg 2005). Different pathways involving distinct effector mechanisms have been suggested to be involved in the pathogenesis, and could possibly explain differences in the extent of demyelination, injury of oligodendrocytes and axonal damage seen among MS patients (Lucchinetti 1996, 2000, 2002). However, most active lesions in patients with long-standing MS are characterised by Igs and complement-mediated phagocytosis of oligodendrocytes and myelin, indicating that the pathogenesis of MS may converge to a common pathway (Breij 2008).

Intrathecal B cell responses in MS

Involvement of Igs in the pathogenesis of MS has long been suspected. Thus, intrathecal synthesis of gamma globulin was first described in 1942, and was shown to be at least partly oligoclonal in 1960 (Kabat 1942; Karcher 1960). An increase in intrathecally synthesised IgG antibodies which often results in oligoclonal IgG bands in the CSF is the most consistent clinical immunological abnormality in MS. Elevated levels of IgG antibodies in the CSF have been thought to be sustained by long-lived plasma cells recruited to or differentiating within the CNS (Prineas & Wright 1978). Extensive replacement mutations in the Ig V gene region of B cells and plasma cells isolated from demyelinated lesions or CSF of MS patients have provided evidence for an antigen-driven intrathecal humoral immune response and that dominant B cell clone populations can persist within the CNS (Qin 1998, 2003; Colombo 2000, 2003; Owens 2003). Post mortem histological findings of brain tissue have revealed structures strikingly similar to B cell follicles containing GCs in the meninges in secondary progressive MS, and found that the presence of such tertiary lymphoid structures are associated with early onset of disease and severe cortical pathology (Prineas 1979; Serafini 2004; Magliozzi 2007). Concordantly, a complete recapitulation of B cell differentiation resembling the GC reaction has been found in the CSF of MS patients (Corcione 2004).

Intrathecal humoral immune responses against microorganisms in MS

Increased IgG antibodies and oligoclonal IgG bands have been detected in the CSF of humans with chronic infectious CNS diseases such as subacute sclerosing panencephalitis, neurosyphilis, cryptococcal, varicella zoster meningoencephalitis and progressive rubella encephalitis (Vandvik 1973; Vartdal 1982; Porter 1977; Vartdal 1982; Coyle 1981). Studies

of the specificity of CSF oligoclonal IgG bands in patients with CNS infections have revealed that the antibodies were directed against the agent that caused the disease (Vandvik 1982; Vartdal 1982). Since the oligoclonal IgG bands in CNS infections are specific for the causative infectious agent, it is conceivable that also the persistent Ig response in MS targets disease-relevant antigens. Despite considerable efforts, the specificities of the main IgG antibody fractions appearing as oligoclonal IgG bands in the CSF of MS patients remains enigmatic.

A peculiar characteristic of MS is the perpetual intrathecal synthesis of virus-specific antibodies (Vartdal 1982). Intrathecal synthesis of specific antibodies against one or more common viruses, such as measles virus (MeV), rubella virus, and varizella zoster virus (VZV) are found in more than 90 % of MS patients (Vartdal 1980; Sindic 1984). Most MS patients display oligoclonal virus-specific IgG bands restricted to the IgG1 subclass upon CSF analysis using IEF with immunoblot (Vartdal & Vandvik 1983). Intrathecal antibody syntheses against MeV and rubella virus are also present in vaccinated individuals with MS (Robinson-Agramonte 2007). Some of these antibodies are directed against RNA-viruses most unlikely to persist within the CNS, suggesting that the virus-specific antibodies are not part of an ongoing response against a latent infection.

The virus-specific IgG antibodies do not correspond to the main oligoclonal IgG bands in the CSF and constitute approximately only 2 % of the total CSF IgG (Reiber 1998). Whereas CSF IgG antibodies from patients with viral encephalitis have been shown to display high binding affinity against the causative agent, intrathecally synthesised virus-specific IgG antibodies from MS patients are of low affinity (Luxton 1995).

Aims of the study

The problems addressed in this study are all related to the dysregulated intrathecal immunity in two chronic neurological disorders, and are grouped under the following subheadings: 1) Virus-specific IgG antibodies produced *in vitro* by CSF B cells from patients with MS and CIS, 2) GAD65-specific IgG antibodies in SPS and 3) GAD65-specific T cells in SPS.

Paper 1: Virus-specific IgG antibodies produced by CSF B cells *in vitro* from MS and CIS patients

Although CSF B cells have been shown to produce virus-specific antibodies *in vitro* (Henriksson 1986; Salmi 1989; Baig 1989), the clonal patterns of these B cells are not known. In this study we wanted to explore:

a) The clonal patterns of total IgG and of virus-specific IgG antibodies against MeV, VZV and herpes simplex virus type-1 (HSV-1) in supernatants of *in vitro* cultures of PBMCs and CSF cells and in sera and CSF from MS and CIS patients.
b) The effect of BAFF on total IgG and on virus-specific IgG production in *in vitro* CSF cultures.

Paper 2: GAD65-specific IgG antibodies in SPS

Earlier studies have shown a persistent intrathecal production of IgG antibodies against GAD65 in SPS (Dalakas 2001; Rakocevic 2004). In this study we wanted to further characterise of GAD65 IgG antibodies in CSF and serum by:

a) Mapping the clonal patterns and subclass distribution of GAD65 IgG antibodies in CSF and serum.
b) Analysing the avidities and binding capacities of serum and CSF derived GAD65 IgG antibodies.
c) Investigating the temporal persistence of clonal patterns of GAD65 IgG in serum and CSF.

Paper 3: GAD65-specific T cells from patients with SPS

Most patients with SPS display systemic and intrathecal production of IgG antibodies against GAD65, but little is known about the mechanisms driving this immune response. In order to explore the intrathecal cellular immune response against GAD65 and compare it with the systemic response, we attempted to clone GAD65-specific T cells from CSF and blood from SPS patients and investigate whether:

a) GAD65-sepcific T cells are present in the CSF and the blood of patients with SPS, and if such T cells are sequestered in the CSF?
b) And if so, what are the HLA restriction and the cytokine profile of GAD65-specific T cells?
c) Also, can we identify the GAD65 epitopes recognised by CSF and blood T cells?

Summary of the papers

Paper 1:

In this paper we showed that *in vitro* cultured CSF cells from all six MS / CIS patients studied, whereas only one of the four control patients, produced oligoclonal IgG. In contrast, *in vitro* production of oligoclonal IgG by PBMCs was not detected in any patient. By using isoelectric focusing (IEF) with immunoblot, *in vitro* cultured CSF cells from all six patients with MS / CIS were shown to produce oligoclonal IgG antibodies against either MeV, VZV or HSV-1. In each patient, the *in vitro* and *in vivo* intrathecally produced antibodies were specific for the same viruses. However, the *in vitro* synthesised total IgG and virus-specific IgG antibodies were shown to display different clonal patterns from those produced intrathecally *in vivo*. Addition of BAFF did not affect the amounts or clonal patterns of either total IgG or virus-specific IgG antibodies produced by *in vitro* CSF cells cultures.

Paper 2:

In this article, the avidities and clonal patterns of the GAD65 IgG antibodies in five patients with SPS were studied. By using radioimmunoassay (RIA) analyses we demonstrated that four of five SPS patients displayed intrathecal synthesis of GAD65 IgG antibodies. Intrathecally and systemically produced oligoclonal GAD65 IgG antibodies, mainly of the IgG1 subclass, was found in all five SPS patients by using IEF with immunoblot. The binding avidity of GAD65 IgG antibodies from CSF was more than 10 times higher than in GAD65 IgG antibodies from serum in two of the patients, but did not differ significantly in the remaining three. All patients displayed higher GAD65 IgG antibody binding capacities (Bmax) in serum than in CSF. The oligoclonal GAD65 IgG bands in CSF and serum

persisted for years. These data indicate that a population of potentially pathogenic intrathecal and systemic GAD6-specific B cells or plasma cells persists in the intrathecal compartment in SPS patients.

Paper 3:

This is the first study of GAD65-specific T cells from the CSF of SPS patients. 11 GAD65-specific T cells were cloned from CSF from three out of four SPS patients. In contrast, only one GAD65-specific T cell clone was generated from blood from one of the patients. The GAD65-specific T cells isolated were predominately restricted by HLA-DR. However, two T cell clones isolated from CSF of one of the patients studied displayed HLA-DP restriction. Mapping of GAD65 T cell epitopes showed that CSF T cell clones recognised four different GAD65 epitopes that were unique to each patient. In two patients, T cells recognising the same GAD65 epitope in the context of identical HLA molecules were cloned from more than one aliquot of the same CSF sample, suggesting that these T cells belonged to the same or to closely related clones, and that they had been clonally expanded *in vivo*. The T cell clone derived from blood recognised a unique GAD65 epitope which differed from the epitopes recognised by the CSF T cells. Notably, cysteine in amino acid position 474 was critical for recognition of GAD65 (474-484) by the HLA-DP restricted CSF T cells isolated from one of the SPS patients. All GAD65-specific T cell clones displayed a predominant Th1 phenotype, but some clones also produced Th2 cytokines. These results suggest that clonally expanded GAD65-specific T cells exist intrathecally in patients with SPS.

Methodological considerations

Patients, sample collection and ethical considerations

Patients

In paper I, MS, CIS and control patients were recruited to the study during routine diagnostic examination at the Department of Neurology, Oslo University Hospital Ullevål by neurologists working there.

In paper II and III, SPS patients were recruited from the Departments of Neurology at Oslo University Hospital Ullevål and Rikshospitalet. The latter serves as referral hospital for rare neurological disorders in Norway. Even though SPS is a rare disease with an estimated prevalence of 1 per 1.000.000, we managed to recruit five and four patients with SPS to the studies comprising paper II and III, respectively. Requests to other neurological departments in Norway have not identified additional patients with SPS and GAD65 IgG antibodies. The SPS patients studied therefore probable represent almost the entire Norwegian population of patients diagnosed with SPS.

In paper II, control patients with neurological diseases were recruited during routine diagnostic workup at the Department of Neurology at Oslo University Hospital Ullevål. In addition, sera from T1D patients with high levels of GAD65 IgG antibodies served as additional controls. These were made available from the biobank at the Hormone Laboratory at Aker University Hospital.

A limitation of paper III is the lack of controls. It would be beneficial to test if it was possible to generate GAD65-specific T cells from CSF or blood of control patients. This was not performed because CSF sampling and repeated blood sampling impose a substantial

burden on the patients, and patients not having SPS would likely be less eager to participate or more easily withdraw from the study.

Sample collection

In order to avoid confounding imposed by changes in the lymphocyte repertoire, paired blood and CSF samples were always collected within less than one hour and cultured in parallel. No visible contamination of blood was recorded during the spinal tap. Nevertheless, the first 2 ml of CSF was always discharged, as even diminutive amounts of blood would render the CSF sample non-representative primarily due to the high levels of cells in blood.

To obtain detectable IgG quantities by *in vitro* cultures of CSF B cells, only MS, CIS and control patients with CSF cell counts ≥ 10 cells per µl were included in paper I. In order to be able to generate several T cell lines from each SPS patient, GAD65-reactive T cell lines were generated from approximately 26 ml CSF (paper III).

Ethical considerations

All work included in this thesis has been approved by the Committee for Research Ethics and all other relevant institutions, and written informed consent was obtained from all patients before they entered the study.

It has been reported in a previous study that post puncture headache (PPH) is common and occur in 46 % of females and in 21 % of males (Vilming 2001). PPH was considered the major torment of using CSF in this study. It has been reported that PPH is more prevalent with the use of a 20- compared with a 22-gauge needle (Vilming 2001). Thin needles were

therefore used throughout. SPS patients were recruited for the study via experienced neurologists who stayed in contact with the patients during the study period.

Antigens

Microbial antigens

As > 90 % of all MS patients has local synthesis against MeV and / or VZV, accordingly these viruses were chosen as antigens (Vartdal 1980). HSV-1 was included as 1 / 3 of MS patients have local production against this virus (Vartdal 1980).

The MeV and VZV antigens were produced from tissue culture of human cells infected with the viruses, whereas HSV-1 antigen was produced from permanent simian kidney tissue culture infected with HSV-1. The lyophilised virus antigens were dissolved in distilled water, sonicated and stored at -70°C. The advantage of using crude cell extracts for coating is that these antigen preparations contain a high range of antigenic determinants (Vartdal 1980). A possible disadvantage could be that they also contain contaminants from the cells and growth medium. However, no cross-reactivity was observed between virus-specific oligoclonal IgG bands from the same patients (paper I). Furthermore, *Borrelia burgdorferi* strain 152 was cultured in our laboratory.

GAD65 protein and GAD65 peptides

Recombinant human GAD65

To analyse the clonal patterns of GAD65 IgG antibodies and select for GAD65-specific T cells from blood and CSF, rhGAD65 was used as antigen. This antigen was expressed in baculovirus infected *Spdoptera frugiperda* cells and the purity was tested to be ≥ 95 % on

sodium dodecyl sulphate polyacrylamide gel electrophoresis (according to the manufacturer), and as well confirmed by high-performance liquid chromatography in our laboratory. As T cells are capable of responding to very low concentrations of antigens, even minute contamination may be sufficient to trigger proliferation of T cells that are specific for the contaminant (paper III). To confirm the specificity of GAD65-reactive T cells, a rhHLA-DQ2 protein was included as a negative control protein (paper III). This negative control protein was produced in the same expression system as rhGAD65 and should therefore contain the same contaminations.

GAD65 peptides

To identify T cell epitopes and to further confirm the specificity of GAD6- reactive T cell clones, a panel of 144 16-mer overlapping peptides (approximately 50 % pure) spanning the complete amino acid sequence of GAD65 protein was synthesised. Each peptide overlapped the consecutive peptide with 12 amino residues, thereby allowing most potential $CD4^+$ T cell epitopes to be represented in the panel. The solubility of the lyophilised peptides was variable, and the peptides were therefore initially dissolved in 100 % dimethyl sulfoxide (DMSO). Still, some of peptides tended to aggregate after dilution in the culture medium. DMSO is toxic. However; the concentration of DMSO in cell culture medium was always kept below the toxic concentration (0.1 %). For confirmation of specificity and determination of minimum epitopes, purified GAD65 peptides, including peptides that were truncated and contained amino acid substitutions, were purchased from another manufacturer (91-100 % pure).

The peptide yield was measured in four control peptides, which were synthesised in addition to the 144 peptides GAD65 peptides comprising the peptide panel. The yield of the

GAD65 peptides was estimated from the yield of the four control peptides. To ensure that the concentration of each peptide was sufficient during screening experiments, a high and an intermediate estimated peptide concentration (100 μM and 10 μM) was used for this purpose. Yet, we cannot exclude that some of the peptide could be present in toxic concentrations or that some peptides were more or less insoluble and therefore present in too low concentrations to be detected by the T cells.

Searching for GAD65-specific T cells in SPS patients

GAD65-reactive T cell lines from CSF and blood were cultured in parallel following identical procedures to minimise the differences imposed by *in vitro* manipulations. In order to select and expand GAD65-specific T cells, T cells from CSF and blood were stimulated with irradiated autologous PBMCs that had been preincubated with rhGAD65 overnight. To select for $CD4^+$ T cells that responded to primary antigen stimulation, IL-2 was not added until day seven of cell culture. Furthermore, as activated T cells express high affinity IL-2 receptors, addition of IL-2 at day seven would provide an extra proliferation signal to GAD65-specific T cells that had received antigen stimulation at day one. The cells were further stimulated at day 16 with irradiated autologous PBMCs that had been preincubated with rhGAD65 overnight. The advantage of repeated antigen stimulation is that specific T cells are selectively and efficiently propagated (Holmøy 2004). To avoid propagation of allospecific T cells, GAD65-specific T cells from CSF and blood cells were cultured in 10 % autologous serum the first 24 days. Moreover, antigen responsive T cell lines were cloned by limiting dilution by seeding 0.1 - 3.0 cells per well. The cloning frequency was 1 - 20 %,

which makes it more than 95% likely that each T cell clone is monoclonal (Fitch & Gajewski 1997).

The main obstacle to the study of CSF cells is, in addition to the before mentioned discomfort for the patients, the limited number of cells that can be obtained from each patient. It is therefore hardly possible to study primary T cell responses in CSF, and the T cells had to be subjected to *in vitro* expansion. An important consideration that must be taken into account is that the *in vitro* milieu may have altered the cytokine profile of the T cells or favoured the expansion of particular subsets of T cells. Although cytokines known to select for particular subsets of $CD4^+$ T cells were not used in the cell protocol, and although we were able to expand both T_H1 and T_H1 / T_H2 polarised GAD65-specific T cell clones from SPS patients, it cannot be excluded that the method used to clone and expand the GAD65-specific T cells may have influenced their cytokine profile. A further challenge is therefore to establish whether the GAD65-specific T cells also produce T_H1 and T_H1 / T_H2 phenotype *in vivo*. Moreover, we have been able to establish both T_H1 and T_H2 polarised glatiramer acetate (GA)-reactive $CD4^+$ T cell lines and clones from both blood and CSF from MS patients by using the same protocol, and also demonstrated that T_H2 polarized GA-reactive T cells accumulate in the CSF (Hestvik 2008). Furthermore, by using a similar method, *Mycobactrium avium* subspecies paratuberculosis-reactive $CD4^+$ T cells with a T_H1 and a T_H1 / T_H17 phenotype have been cloned from intestinal biopsies of Crohn's disease patients (Olsen 2009). Collectively, these data indicate that the *in vitro* procedure used to clone $CD4^+$ T cells in paper III is well suited for propagation of different phenotypes of $CD4^+$ T cells.

Irradiated autologous PBMCs were used as APCs in all T cell assays. As repeated antigen stimulation was used to establish GAD65-reactive T cell lines, it is particularly important to consider whether such APCs also stimulate naïve T cells, that are likely less relevant for the

disease process. PBMCs contains several potential APCs, including monocytes, B cells and some DCs. Notably, a previous study revealed that while both naïve and memory T cells from both patients with primary biliary cirrhosis and healthy subjects proliferated upon stimulation with antigen-loaded autologous PBMCs, whilst only memory T cells from the primary biliary cirrhosis patients responded to antigen-loaded costimulation-incompetent APCs (Shimoda 2008). It is therefore possible that the cell culture procedure applied in this work also propagated some naïve GAD65-specific T cells. This is, however, most relevant for the blood-derived T cell lines, as predominantly activated T cells are supposed to enter the CNS (Hickey 1991). The T cells present in CSF are therefore likely to be of an activated phenotype, whereas T cells with a naïve phenotype that are less relevant for the disease constitutes a larger proportion of the T cells present in the blood.

T cell proliferation assays and analysis of cytokine production

In this study, T cell proliferation was measured by a [^{3}H] thymidine incorporation assay (paper III). This is a robust assay and well suited for screening and monitoring large number of T cell lines and clones. However, this method selects for T cells that proliferates upon activation, and neglects antigen-specific T cells with less pronounced proliferative potential, such as regulatory T cells. Another disadvantage of this method is the handling and disposal of radioisotopes, and it is therefore performed according to strict procedures.

The cytokine secretion was measured in cell culture supernatants by employing a bead-based multiplex system using Luminex 100 technology (paper III). Supernatants from T cells stimulated or not and *in vitro* CSF B cell cultures were harvested after 48 hours and seven days respectively, and kept frozen at - 70°C until analysis. In this assay, several populations

of beads in suspension are linked to different unique antibodies that can be separated from each other based on internal fluorescent dyes. The advantage of this assay is that a large number of samples can be analysed simultaneously for the presence of multiple cytokines.

B cell culture

To examine the clonal patterns of IgG and virus-specific IgG antibodies produced by *in vitro* cultured blood and CSF cells, supernatants from short term B cell cultures were studied. After collection of CSF, the cells were isolated by centrifugation. To prevent cell loss, CSF cells were washed only once in culture medium before cultivation. The blood was obtained within 30 minutes of the lumbar puncture, and PBMCs and CSF cells were cultured for seven days before the cell-free supernatants were analysed for IgG. In pilot experiments, CSF cell cultures from patients with ≤ 10 cells / µl did not secrete detectable amounts of IgG (detection limit 0.1 mg / ml) as measured with nephelometry. A short-coming of the nephelometry assay is that IgG concentration below 10 mg / ml is not always very reliable, however the measured *in vitro* synthesised IgG concentration correlated well with corresponding CSF and serum IgG when analysed on IEF for total oligoclonal IgG (paper I).

Detection of IgG and IgG antibodies

Quantification of GAD65 IgG by radioimmunoassay

To quantify GAD65 IgG in sera and CSF from SPS and control patients we have used RIA, which is a simple, reproducible and standardised assay that is routinely performed at the Hormone Laboratory at Aker University Hospital. Briefly, RIA was performed by using *in vitro* transcribed and translated [^{3}H]-Leucine labelled humane islet GAD65 (1.8 x 10^{7} dpm /

pmol) performed in *Escherichia coli* (*E. coli*) (Petersen 1994). Samples were incubated overnight with radio labelled GAD65, followed by isolation of immunocomplexes and counting of [^{3}H]-Leucine GAD65 in a Microbeta counter. In order to quantify intrathecal GAD65 IgG synthesis, serum IgG samples were diluted to the same total IgG concentration as in the corresponding CSF sample, and GAD65 IgG activity was then measured in serial dilutions of the CSF and adjusted serum pair. The CSF / serum GAD65 IgG ratio was calculated from the activity in CSF and serum at three different dilutions, corresponding to the most linear part of the titrations curves.

The main advantage of RIA is that the immunoreactions occur in free solution, meaning that there should be an unrestricted access for antibodies to antigenic epitopes. A potential disadvantage is that the *in vitro* translated GAD65 may not display identical antigenic properties as GAD65 expressed in neurons *in vivo* or in mammalian *in vitro* expression systems, because no posttranslational modifications take place in prokaryote cells such as *E. coli* (Petersen 1994).

Qualitative analysis of IgG and IgG antibodies

The detection of intrathecal synthesis of oligoclonal IgG with IEF is a useful diagnostic tool both in MS and other disorders of the CNS (Deisenhammer 2006). IEF with immunoblot was used to examine the clonal patterns of intrathecally synthesised IgG and of virus-specific IgG antibodies in supernatants of *in vitro* cultures of PBMCs and CSF cells and in sera and CSF from MS and CIS patients (paper I). Additionally, this technique was employed to study the clonal patterns of GAD65 IgG antibodies in sera- and CSF-pairs from SPS patients and sera from T1D patients (paper II). IEF with immunoblot uses a pH gradient to separate IgG populations on the basis of their charge. The isoelectrofocused proteins were then blotted

onto membranes with or without antigen, and visualised with enzyme-coupled anti-IgG antibodies. IEF is sensitive for detection of intrathecally synthesised IgG and can be performed on low amounts of IgG (paper I). However, the technique is even more sensitive when antigen coated membranes are used to detect synthesis of oligoclonal antigen-specific IgG antibodies as such antibodies are not ′drowned′ by the background of total IgG (Deisenhammer 2006; Vartdal 1980).

A potential drawback of this technique is that when the proteins are immobilised by electrostatic polarity and Van der Waals forces on the nitrocellulose membrane, steric hindrance may influence antibody access to antigenic epitopes (Vianello 2005). Using RIA and IEF with immunoblots, high GAD65 IgG antibody activity and oligoclonal bands were detected in sera and CSF in all patients with SPS. In contrast, high GAD65 IgG antibody activity was detected by RIA in sera from T1D patients, but no oligoclonal GAD65 IgG bands (paper II). These results may be attributed to the well described differences in target epitopes of GAD65 IgG antibodies between neurological and diabetes patients (Daw1996).

Avidity and binding capacity of GAD65 IgG antibodies

In our study we employed Scatchard analysis to determine the K_D and Bmax of GAD65 IgG antibodies from SPS and T1D patients (McPherson 1985). In this competition assay, displacement of [^{3}H]-Leucine GAD65 binding to GAD65 IgG antibodies in serum or CSF samples by unlabeled GAD65 was performed by adding 0 - 15 pmol unlabeled GAD65. Binding data were analysed by non-linear curve analysis according to a one binding site model using the computer program KELL (McPherson 1985). Noteworthy, Scatchard analyses is not extremely reliable and the values for K_D can have high margin of error (McPherson 1985). Binding studies of antibodies can be carried out by using surface plasmon resonance

technique. Although binding studies of antibodies can be carried out by using surface plasmon resonance technique, Scatchard analysis is, however, still commonly used (Drake & Klakamp 2007).

General discussion

The main general topics in this discussion are: 1) the relevance of CSF lymphocytes for the study of the immunopathogenesis of MS and SPS, 2) autoimmunity to GAD65 in SPS, and 3) B cell involvement in the intrathecal synthesis of virus-specific IgG antibodies in MS and GAD65 IgG antibodies in SPS.

The relevance of CSF lymphocytes in the studies of MS and SPS

From experimental models, in particular EAE, it has become clear that immune responses within the CNS have specific characteristics compared with systemic immunity (Steinman 2001). In humans, the compartmentalisation of the immune response in CNS is underscored by the perpetual intrathecal synthesis of oligoclonal IgG in MS, CNS infections and other immune mediated neurological diseases, including SPS (Owens 2006; Dalakas 2001). CSF T and B cells from MS patients have been reported to be clonally expanded (Oksenberg 1990; Wucherpfennig 1992; Qin 1998; Colombo 2000; Owens 1998, 2003; Obermeier 2008). As the CSF is contiguous with the extracellular fluid of the CNS, analysis of distribution and phenotype of inflammatory cells in the CSF may help us to understand the unique immunological conditions within the CNS compartment.

Immune surveillance of the subarachnoid space is not necessarily identical with that of the CNS, and whether the IgG production from CSF cells reflects the humoral immune response in the CNS is unknown. In support of the relevance of CSF B cells, it has recently been shown that transcribed V(D)J genes from CSF B cells correspond with the amino acid sequence of CSF Ig (Obermeier 2008). Although the specificity of the main fractions of the

oligoclonal IgG is unknown, intrathecal synthesis of oligoclonal IgG antibodies against a broad panel of viral agents including MeV, VZV and HSV-1 is a characteristic feature of MS (Vartdal 1982). Comparison of the virus-specific IgG in the CSF with that produced *in vitro* by CSF B cells therefore offers a possibility to address whether CSF B cells actually reflect the IgG-producing B cells in the CNS. In paper I we therefore asked if the clonal patterns and specificities of antibodies to MeV, VZV and HSV-1 produced *in vitro* by CSF cells from MS and CIS patients correspond to that produced intrathecally *in vivo.* We showed that *in vitro* cultured CSF cells from these patients produced oligoclonal IgG antibodies against the same viruses targeted by oligoclonal CSF IgG antibodies synthesised intrathecally *in vivo.* Although the overall numbers of virus-specific IgG bands and their clonal patterns differed, the results indicate that the specificities of antibody producing B cells in the CSF reflect those of antibody synthesising cells in other locations of the CNS.

It has been suggested that IgG-mediated complement deposition within MS lesions plays an important role in the pathogenesis of the disease (Breij 2008). This might suggest that the concentration of the most relevant antibodies is reduced in the CSF, as these antibodies may be bound in the MS lesions, or that the antibodies produced from CSF cells represent only a fraction of a much larger repertoire of antibody producing cells within the CNS. Likewise, the most relevant B cells might be trapped in the tissue, whereas B cells present in the CSF might be irrelevant bystanders. B cell clones producing low-avidity virus-specific IgG antibodies could possibly be an example of such irrelevant bystanders, as several of the viruses targeted by these IgG antibodies are not likely to be present in the CNS. Nevertheless, the corresponding specificities between virus antibodies in CSF and *in vitro* CSF B cell cultures highlight the relevance of studying CSF B cells in MS and possibly also in other

neurological diseases. The results do, however, not prove that CSF B cells correspond with B cells present within the CNS lesions or elsewhere in the intrathecal compartment.

One of the major issues in understanding the pathophysiology of human autoimmune diseases is to identify the target antigens that drive the clonal expansion of autoreactive T cells. In paper II we showed that the synthesis of GAD65 IgG antibodies in SPS is oligoclonal and mediated by a stable population of affinity maturated B cell clones, suggesting that these antibodies are produced by B cells having received T cell help. Indeed, in paper III, we found evidence suggesting that HLA-DR or HLA-DP restricted GAD65-specific $CD4^+$ T cells accumulate in the CSF of SPS patients with intrathecal synthesis of GAD65 IgG antibodies. Also, in two of the patients studied, we found evidence suggesting that identical or closely related GAD65-specific CSF $CD4^+$ T cell clones had been expanded *in vivo*. In contrast, only one GAD65-specific T cell clone could be raised from the blood in one of the patients, implying that GAD65-specific T cells from SPS patients had accumulated in the vicinity of the diseased organ.

These results concur with those from other immune mediated diseases, in particular celiac disease, showing that disease relevant T cells accumulate in the diseased organ (Molberg 1998). Notably even though disease-specific T cells from patients with celiac disease and T1D can enter the circulation for short periods of time, it has been difficult to identify clonally expanded disease specific-T cells in peripheral blood (Hafler 1988; Raki 2007). Furthermore, although a lack of a systemic response to insulin peptides in T1D patients, expanded T cells recognising an insulin epitope have been observed in draining pancreatic lymph nodes (Kent 2005). Together with the findings presented in this thesis, these observations support the thought that T cells present in the diseased organ more accurately

reflect the cell population relevant for the disease process. This question has been difficult to address in MS and most other immune mediated CNS diseases, because the specificity of the immune response is not established. In this context it is also noteworthy that a previous study of 36 MS patients showed an overrepresentation of $CD8^+$ T cells expressing particular TCR-variable β chains in the CSF compared with the blood in the majority of patients, suggesting that clonal T cell expansion occurring in the CSF can be detected in MS patients (Jacobsen 2002). These results concur with the observations on GAD65-specific T cells in SPS presented in paper III.

Autoimmunity to GAD65 in SPS

Do GAD65 antibodies or any of the other SPS-associated autoantibodies described to date cause SPS, or are they only markers of autoimmunity? One well-rehearsed argument is that SPS autoantibodies are not pathogenic, because they are directed against intracellular proteins (Duddy & Baker 2009). This question is, however, beyond the scope of this thesis and will therefore not be further discussed. Some of the findings in this thesis may however have implications for the understanding of the immunobiology of GAD65 both in SPS and in more general terms.

The synthesis of GAD65 IgG antibodies

Even though T - B cell collaboration has not been studied directly in this thesis, the data in paper II and III indicating that clonally expanded GAD65-specific T cells co-exist with clonally expanded GAD65-specific B cells in the diseased organ, suggest a role for T cells in sustaining the intrathecal synthesis of GAD65 IgG antibodies in SPS. The exact anatomical

location for the intrathecal synthesis of GAD65 IgG antibodies in SPS remains unknown. Recently, one of the SPS patients studied in this thesis (patient SPS 3) has deceased. His symptoms included stiffness and spasms typical of SPS, as well as a malignant course with lower motor signs in one leg compatible with PER. This patient displayed intrathecal synthesis of oligoclonal GAD65 IgG antibodies as well as clonal expansion of GAD65-specific T cells. Autopsy revealed unilateral axonal swelling, chromatolysis and vacuolisation of anterior horn cells of the lower spinal cord, in addition to discrete infiltration of $CD8^+$ cytotoxic T cells and microglial proliferation, but no $CD4^+$ T cells, B cells or plasma cells were detected within the inflamed section of the spinal cord or elsewhere in the CNS (Holmøy 2009). Obviously, this does not exclude that such cells were present in other locations of the CNS than those that were subjected to neuropathological examination. The meninges have to my knowledge not been examined in autopsies from SPS patients, and may be an important localisation for the intrathecal synthesis of GAD65 IgG. The lack of $CD4^+$ T cells, B cells and plasma cells within the inflamed section of the spinal cord may imply that the intrathecal synthesis of GAD65 IgG antibodies is not directly related to the inflammation associated with tissue destruction (Holmøy 2009). Thus, GAD65-specific T - B cell collaboration – if it occurs – rather takes place elsewhere, either in the meninges or in other places inside or outside of the CNS.

Interestingly, as shown in papers II and III, we were not able to clone GAD65-specific T cells from CSF from the only SPS patient (SPS 1) without substantial intrathecal synthesis of GAD65 IgG antibodies. Given that this patient displayed high GAD65 IgG antibody activity and binding capacity in serum, we speculate that physiological transfer of GAD65 IgG antibodies from serum to CNS may be sufficient to evoke neurological symptoms (paper II). Accordingly, many patients with voltage-gated potassium channel (VGKC) antibodies and

immunotherapy-responsive limbic encephalitis do not have oligoclonal IgG bands and their CSF is negative for VGKC antibodies (Vincent 2004; Jarius 2008).Thus, the lack of an intrathecal GAD65 IgG antibody synthesis does not necessarily exclude autoimmunity.

GAD65 as an autoantigen

An enduring question in autoimmunity is why only a limited proportion, ~ 2-3 %, of all human proteins becomes selected as autoantigens (Plotz 2003). It is striking that most autoantigens are localised intracellularly, raising the question of why they are targeted and the consequences of the immune response (Fenalti & Rowley 2008). The recent publication of N-terminally truncated crystal structures for the two isoforms, GAD65 and GAD67, have revealed that only the former has a very flexible and inherently mobile sequence in the C-terminal region, which may render this region highly available to endopeptidases (Fenalti & Rowley 2008). Strikingly, non-obese diabetic (NOD) mice exhibit a spontaneous proliferative response to GAD65 at the onset of insulitis (Kaufman 1993; Tisch 1993). The T cell responses are initially directed towards a few so-called spontaneous epitopes (residues 509-528 and 524-543 in the C-terminal, region of the GAD65 molecule) with later spreading to other T cell epitopes as the disease progresses (Kaufman 1993; Dai 2005). Interestingly, GAD65-specific T cell epitopes from one of the SPS patients (SPS 2) were localised within the C-terminal region (residues 474-484 and 555-565) of GAD65. This is in line with previous findings in T1D showing that T cell epitopes to GAD65 localise to residues 481-495, 511-525 and 551-585 (Patel 1997; Nepom 2001). Furthermore, the C-terminal region is also targeted by $CD8^+$ T cells and of interest, tolerance-inducing DNA coding for the C-terminal region, $GAD_{500-585}$, has been shown to protect NOD mice from diabetes (Quinn 2001; Han 2005).

Furthermore by combining the N-terminally truncated high-resolution crystal structure of GAD65 with an "immunological" panel of 11 monoclonal antibodies to GAD65 established from T1D patients, the location of antibody epitopes on GAD65 has been examined (Fenalti 2008). Two mutually exclusive clusters of B cell epitopes have been defined on opposing faces of the C-terminal domain of GAD65 in T1D (Fenalti 2008). Notably, T cell epitopes from T1D patients are localised to the same surface region of GAD65 (Fenalti & Rowley 2008). Of interest, T and B cell epitopes from T1D patients overlap on GAD65, but the immunological meaning of this remains to be understood. While antibodies to GAD65 from T1D patients recognise mainly conformational epitopes, additional linear epitopes on GAD65 are recognised in SPS (Lernmark 1996). Particularly, an N-terminal linear epitope that distinguishes sera from SPS and T1D has been repeatedly reported (Butler 1993; Kim 1994; Raju 2005). None of the GAD65-specific T cell epitopes from the SPS patients studied, paper III, overlapped with the identified linear N-terminal GAD65 antibody epitope (residues 4 - 22). As no monoclonal antibody to GAD65 has been established from SPS patients until now, it cannot be excluded that the GAD65 T cell epitopes may overlap with other B cell epitopes to GAD65 characteristic for SPS. This question also needs to be addressed in SPS, to provide more insights into the GAD65 immunobiology.

Breaking tolerance to GAD65

Although a number of mechanisms have been proposed and contributing genes have been identified, the cause of autoimmunity is still elusive. As in most autoimmune diseases, the factors initiating loss of tolerance to GAD65 in SPS are not clear.

In paper III we report two DP-restricted CSF T cell clones that initially responded weakly to rhGAD65 protein but later failed to respond to several batches of rhGAD65 protein. These

clones responded vigorously and persistently to synthetic GAD65 peptides, residues 474-484, showing that the failure to recognise rhGAD65 protein was not due to loss of responsiveness or specificity. Although we cannot exclude difference in quality between the different rhGAD65 protein preparations, it is peculiar that this epitope carries a cysteine residue (C_{474}) found to be critical for T cell recognition. The six cysteine residues located in the N-terminal domain of GAD65 have been shown to form disulfide bonds *in vitro* (Battaglioli 2005). The C_{474} residue may be involved in a disulfide bond that is structural protected, and may therefore potentially be part of a cryptic epitope that has not been presented to T cells during T cell education in thymus. Under normal physiological conditions, cryptic epitopes are inefficiently processed and presented (Sercarz 1993). However, under inflammatory and other immunostimmulatory conditions, upregulation of antigen processing events can lead to enhanced processing, including enzymatic cleavage of disulfide bonds, and presentation of the cryptic epitopes by the APCs (Lehmann 1992). This may lead to the priming of cryptic epitope-specific T cells. Notably, the GAD65-specific T cells (paper III) produced substantial amounts of IFN-γ, which among other functions could induce expression of IFN-γ dependent proteases within the CNS. Moreover, further studies must be conducted to determine if oxidation of cysteine indeed is the cause of an inefficient processing of the GAD65 epitope. It is, however, an intriguing possibility that the relatively cryptic GAD65 epitope, residues 474-484, has been unmasked by intrathecal APC during the diseases process of SPS, and therefore made available for intrathecal T cells.

In paper II we showed that the intrathecally and systemically synthesised GAD65 IgG antibodies in SPS generally have high binding avidity, and that the avidity was higher in CSF than in serum in some patients. Yet, how the antibodies can influence antigen processing and

thereby affect GAD65 presentation to T cells in SPS is unknown. One possibility is that GAD65 IgG antibodies bind GAD65, thereby enhancing uptake and presentation of GAD65 by APCs. Numerous studies have shown that soluble monoclonal antibodies can increase presentation of the antigen they bind to by facilitating antigen capture through fragment crystallisable receptors (Ravetch & Bolland 2001). A study using GAD65IgG antibody-positive sera from T1D patients found enhanced presentation of an immunodominant T-cell epitope when GAD65 immune complexes rather than antigen alone were used to stimulate PBMCs (Reijonen 2000). However, it has been observed that high affinity antibodies does not dissociate from antigen at mildly acidic pH of the processing compartment and, therefore, the substrate for proteases is the antigen - antibody complex rather than antigen alone (Lanzavecchia 1995). In APCs antigen-antibody complexes can remain intact after internalisation and fragmentation by proteases along the antigen-processing pathway, thus protecting residues from proteolysis and thereby modulate the presentation of peptides to T cells (Quaratino 2005). Consequently, the fine specificity of a soluble antibody that binds to an antigen can affect the processing and presentation of T cell epitopes by either boosting or suppressing a particular epitope (Simitsek1995; Watts & Lanzavecchia 1993). Interestingly, it has been demonstrated in T1D that GAD65-specific B cells and antibodies can modulate the autoimmune T cell repertoire by down-regulating T cell epitopes in an immunodominat area whilst boosting distant or cryptic regions (Jaume 2002; Banga 2004). Whether the autoimmune GAD65-specific B cells and the antibodies they secrete may play a role in shaping the autoimmune T cell responses in SPS have not been elucidated. The results presented in paper II and III do; however, show that some of the requirements for such T-B cell collaboration are present in the intrathecal compartment of patients with SPS. Further studies are, however, required to reproduce this. Understanding how GAD65 antibodies can

influence presentation of T cell epitopes may hold important clues on the mechanisms controlling autoimmunity both in SPS and T1D.

Antibody secreting cells of the intrathecally synthesised virus-specific IgG antibodies in MS and GAD65 IgG antibodies in SPS

The mechanisms of the perpetuating intrathecal synthesis of virus-specific IgG antibodies and GAD65 IgG antibodies in MS and SPS respectively, are not known. It has been shown that the clonal patterns of virus-specific oligoclonal IgG antibodies in MS may be dynamic during early stages of the disease, but that they mainly remain stable (Sandberg-Wollheim 1987). In paper I, we showed that BAFF supplementation did not affect the amount of IgG or clonal patterns of virus-specific IgG antibodies produced by *in vitro* CSF cell cultures, indicating that cells producing these antibodies are of plasma cell phenotypes not affected by BAFF, as only transitional phases of B cells express BAFF-receptors (Dalakas 2008).

Interestingly, it has been observed that anti-CD20 B cell depleting treatment in relapsing-remitting MS patients significantly decreases the number of B cells in peripheral blood and CSF, and in addition depletes B cells from cerebral perivascular spaces, while the intrathecally synthesised oligoclonal IgG bands remain unaffected (Hauser 2008; Stuve 2005; Martin Mdel 2009; Monson 2005). This may indicate that the intrathecal synthesis of oligoclonal IgG antibodies in MS is generated from long-lived plasma cells not affected by the treatment, as CD20 is not expressed on plasma cells. The tendency of the oligoclonal IgG bands to persist in MS patients with anti-B cell depletion therapy may imply that the CNS provides a long-term survival niche for plasma cells producing antibodies that are not directly involved in the pathogenesis (Meinl 2006). It would be interesting to examine the persistence

of intrathecal virus-specific oligoclonal IgG antibodies after anti-CD20 B cell depletion to further understand the role of both these B cell subsets and virus-specific antibodies in MS pathogenesis.

In paper II we reported that class-switched, high avidity GAD65 IgG antibodies in the CSF and serum from SPS patients are oligoclonal. In addition, the pattern of oligoclonal GAD65 IgG bands in CSF and serum in three SPS patients remained unchanged several years after symptom debut. Notably, these patients have not received immunomodulating drugs that could alter the immune response to GAD65. Of interest, a single case report on an SPS patient demonstrated a rapid decline of intrathecal GAD65 IgG antibodies following anti-CD20 treatment, which suggested a successful targeting and elimination of autoantibody producing B cells within the CNS (Baker 2005). Furthermore, clinical trials with B cell depletion in other autoimmune diseases, such as RA and SLE, reported that certain autoantibodies, anti-DNA and rheumatoid factor, decayed after anti-CD20 B cell therapy (Cambridge 2003; Sfikakis 2005; Ahuja 2007). It is tempting to speculate that the continuously produced GAD65 IgG antibodies in SPS patients are being generated rapidly from renewing $CD20^{+}$ B cell precursors, most consistent with the short-lived plasmablasts pathway. This is indeed consistent with our findings of intrathecal GAD65-specific T cells in paper III. Whether GAD65 specific B cells are also involved in antigen-presentation to GAD65-specific T cells is unknown. This may imply that the autoantibody response, although chronic in terms of the individual, may be a dynamic and ongoing process (Shlomchik 2008).

Future perspectives

Human monoclonal GAD65 IgG antibodies from SPS patients

Although the results presented in this thesis show that GAD65-specific T and B cells coexist intrathecally in SPS patient, their role in the pathogenesis remains unknown.

To establish whether epitope-specific GAD65 antibodies have a role in the pathogenesis of SPS will require *in vivo* transfer studies of GAD65 antibodies from SPS patients. As GAD65 antibodies in CSF and serum may have different properties regarding epitope specificity and avidity, and because GAD65-specific antibodies only comprise a small fraction of IgG from serum and CSF, such experiments should ideally be performed with purified CSF and serum antibodies from SPS patients. Unfortunately, it is not likely to be feasible to obtain sufficient quantities of GAD65 antibodies from CSF of SPS patients. Moreover, it would not be very difficult to identify which of the many GAD65 specific IgG clones present in serum or CSF samples that eventually mediate the pathogenic effect. An alternative approach would be to use monoclonal antibodies with distinct epitope specificities. CSF B cells have previously been cloned from the CSF of MS patients in our laboratory (Holmøy 2005). Yet the limited number of antigen specific B cells has so far precluded the cloning of human GAD65 specific CSF B cells.

To approach this issue, we are in the process of establishing a method for making human monoclonal antibodies based on an improved method for EBV immortalisation of human memory B cells (Traggiai 2004). The advantage of EBV-immortalised B cells is to have a continuous and direct source of GAD65-specific antibody secreting and antigen presenting cells. Additionally, B cell lines producing monoclonal GAD65 IgG offering a possibility to characterise monoclonal antibodies on the protein and DNA level to clarify the role of

epitope specificity, affinity maturation and selection of GAD65-specific IgG antibodies in SPS. Moreover, they could be valuable tools for the study of T - B cell collaboration in SPS.

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